

## Effect of Insect Larval Midgut Proteases on the Activity of *Bacillus thuringiensis* Cry Toxins<sup>▽</sup>

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To test the possibility that proteolytic cleavage by midgut juice enzymes could enhance or inhibit the activity of *Bacillus thuringiensis* insecticidal toxins, once activated, the effects of different toxins on the membrane potential of the epithelial cells of isolated *Manduca sexta* midguts in the presence and absence of midgut juice were measured. While midgut juice had little effect on the activity of Cry1Aa, Cry1Ac, Cry1Ca, Cry1Ea, and R233A, a mutant of Cry1Aa from which one of the four salt bridges linking domains I and II of the toxin was eliminated, it greatly increased the activity of Cry1Ab. In addition, when tested in the presence of a cocktail of protease inhibitors or when boiled, midgut juice retained almost completely its capacity to enhance Cry1Ab activity, suggesting that proteases were not responsible for the stimulation. On the other hand, in the absence of midgut juice, the cocktail of protease inhibitors also enhanced the activity of Cry1Ab, suggesting that proteolytic cleavage by membrane proteases could render the toxin less effective. The lower toxicity of R233A, despite a similar in vitro pore-forming ability, compared with Cry1Aa, cannot be accounted for by an increased susceptibility to midgut proteases. Although these assays were performed under conditions approaching those found in the larval midgut, the depolarizing activities of the toxins correlated only partially with their toxicities.

After having been ingested by susceptible insect larvae, *Bacillus thuringiensis* toxins become soluble in the midgut lumen and activated by partial proteolysis. The activated toxins bind to specific receptors and form pores in the apical membranes of the midgut epithelial cells. These pores abolish ionic gradients and cause lysis of the epithelial cells, leading to death of the insect (33). In vivo, diet composition affects the insect midgut microenvironment (34) and toxin potency (1–4, 6, 8, 15, 18, 25, 26, 28, 32, 35). In vitro, previous studies have demonstrated differential effects of pH (11, 36), ionic strength (11), and divalent cations (11) on the activity of these toxins. However, adjusting these factors so that the experiments were performed under conditions closer to those found in the midgut microenvironment did not allow a perfect correlation between the activities of these toxins measured during in vitro experiments (11) and their in vivo toxicities (40, 41).

Another factor that could be a determinant of toxin activity is the presence of proteases in the midgut microenvironment. Numerous potential proteolytic cleavage sites within the activated toxin have been reported (16). Further cleavage could either enhance or inhibit toxin activity. Lightwood et al. (20) have suggested that cleavage of Cry1Ac by midgut proteases, after its binding to the receptor, could facilitate its insertion into the membrane and accelerate pore formation. Furthermore, Gómez et al. (13) proposed that proteolytic cleavage of helix  $\alpha 1$  in domain I of Cry1Ab, allowed by the binding of the toxin to its cadherin-like receptor, could be essential for pore formation. However, protease inhibitors failed to prevent or even reduce the rate of pore formation by Cry1Aa in brush

border membrane vesicles, suggesting that membrane proteases do not contribute to pore formation by the trypsin-activated Cry1Aa (16). On the other hand, Coux et al. (7), who have introduced mutations in Cry1Aa and Cry1Ac that eliminate one of the salt bridges linking domains I and II, have suggested that the lower toxicity of these mutants, including the Cry1Aa mutant R233A, despite a level of pore formation in midgut brush border membrane vesicles which was comparable to those of Cry1Aa and Cry1Ac, could be attributable to their increased susceptibility to proteolysis in the insect midgut.

In the present study, the effect of midgut juice on the activity of trypsin-activated Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, Cry1Ea, and R233A in isolated midguts of *Manduca sexta* was therefore investigated using membrane potential measurements (11, 30). Midgut juice had a strong effect on the activity of Cry1Ab, but this effect cannot be attributed to proteases. On the other hand, although these experiments were performed in the presence of several factors characteristic of the midgut environment and known to affect toxin activity (11, 36), including a highly alkaline pH, a high ionic strength, and the presence of calcium ions, toxin activity correlated only partially with toxicity both in the presence and absence of midgut juice.

### MATERIALS AND METHODS

**Chemicals.** A cocktail of protease inhibitors containing 50 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.65 mM antipain, 0.015 mM aprotinin, 0.1 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, and 0.1 mM leupeptin was used at a 100-fold dilution. All chemicals were purchased from Sigma (St. Louis, MO).

**Insects.** Fertilized *M. sexta* eggs were obtained from the North Carolina State University Entomology Department insectary (Raleigh, NC). Larvae were reared on a standard synthetic medium supplied with the insects.

**Toxins.** The Cry1Aa mutant R233A was created by oligonucleotide-directed in vitro mutagenesis (7). The wild-type toxins Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, and Cry1Ea and the R233A mutant were prepared from *B. thuringiensis*

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strains producing the appropriate single recombinant toxins as described elsewhere (23). Protoxins were made soluble and trypsin activated (22). Activated toxins were purified by fast protein liquid chromatography using a Mono Q ion-exchange column (Pharmacia Biotech, Montreal, Qc, Canada). Bound toxin was eluted with a 50 to 500 mM NaCl gradient in a 20 mM sodium carbonate buffer (pH 10.8) (22).

**Bioassays.** Toxicity assays were performed on neonate larvae with activated toxins as described previously (7). Toxins were diluted in phosphate-buffered saline (8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, and 150 mM NaCl [pH 7.4]). Toxin samples (100 µl) were layered onto 1.82-cm<sup>2</sup> wells and allowed to stand until the liquid was completely absorbed by the 6-mm-thick medium. To measure the 50% lethal concentration (LC<sub>50</sub>), Cry1Aa, Cry1Ab, and Cry1Ac were tested at 9 concentrations ranging from 200 to 3,200 ng/ml (11 to 175 ng/cm<sup>2</sup>), Cry1Ca and Cry1Ea at 12 concentrations ranging from 400 to 4,800 ng/ml (22 to 253 ng/cm<sup>2</sup>), and R233A at 5 concentrations ranging from 1,000 to 5,000 ng/ml (55 to 274 ng/cm<sup>2</sup>). To evaluate their effect on the weight gain of the larvae, toxins were also tested at nine concentrations ranging from 10 to 1,000 ng/ml (0.55 to 55 ng/cm<sup>2</sup>) for Cry1Aa, Cry1Ab, and Cry1Ac and at seven concentrations ranging from 200 to 2,000 ng/ml (11 to 110 ng/cm<sup>2</sup>) for Cry1Ca and Cry1Ea. Each larva was weighed, placed alone in a well, and reared at 26°C and 70% relative humidity with a photoperiod of 12 h light and 12 h darkness. At least six groups of 25 larvae were tested at each toxin concentration. After 7 days, mortality was recorded and surviving larvae were weighed. Data were adjusted for the mortality of control larvae reared in the absence of toxin. LC<sub>50</sub>s and 50% weight gain inhibitory doses were determined by Probit analysis (10).

**Midgut juice.** To isolate midgut juice, whole midguts were dissected from fifth-instar *M. sexta* larvae and their contents emptied into a centrifuge tube. The insoluble material was removed by centrifugation at 7,500 × g for 10 min. Resulting supernatants were aliquoted, stored at -80°C, and used within 2 weeks. Thawed aliquots were conserved on ice for a maximum of 7 h. Potassium and sodium concentrations were measured with an IL-943 flame photometer (Fisher Scientific, Montreal, Qc, Canada), and chloride concentration was measured with a 4-2500 digital chloridometer (Buchler Instruments, Fort Lee, NJ). Osmolality was measured with a Vapro 5520 vapor pressure osmometer (Wescor, Logan, UT).

**Membrane potential measurements.** Experiments were conducted as described previously (11, 30). The standard 32K solution (27) was modified to better reflect the conditions present in the *M. sexta* midgut without changing the osmolality of the solution (11). The perfusion solution (122K) thus contained 122 mM KCl, 5 mM CaCl<sub>2</sub>, and 5 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid)-KOH (pH 10.5). Oxygen gas was bubbled vigorously for 30 min through the solution immediately before use. Fresh midguts were isolated from actively feeding third-instar *M. sexta* larvae and rinsed with the 122K solution. The Malpighian tubules and peritrophic membrane, with its food content, were removed with forceps. Isolated midguts were aspirated into a glass pipette until their ends curled over the pipette tip, thus exposing the luminal surfaces of their epithelial cells (30). Midgut cells were impaled through their apical membranes with a glass microelectrode filled with 1 M KCl. Electrode resistance was between 100 and 200 MΩ. The signal was amplified with a KS-700 microprobe system apparatus (WP Instruments, New Haven, CT) and recorded on a strip chart recorder (Houston Instruments, Austin, TX). The bath was perfused with the 122K solution at approximately 1 ml/min until the membrane potential was stable over 5 min. Perfusion was then stopped, and 1 ml of perfusion solution containing one of the toxins was added directly to the bath. After 5 min, the preparation was rinsed with the toxin-free perfusion solution for 10 min. All experiments were carried out at room temperature (22 to 24°C).

In some experiments, 10% (vol/vol) midgut juice, 10% (vol/vol) midgut juice that had been boiled for 5 min, the protease inhibitor cocktail, or the protease inhibitor cocktail and 10% (vol/vol) midgut juice were added during the 5-min incubation period with the toxin. In all experiments, the bathing solution was adjusted to maintain the potassium concentration equal to that present in the 122K solution. Membrane potential was normalized by dividing experimental values by the average of the initial membrane potentials (*V*<sub>0</sub>) measured over the first 5 minutes. PR<sub>65</sub> is defined as the time necessary to reduce membrane potential by 65% after the toxin is added to the bath. Statistical analyses were done using the two-tailed unpaired Student *t* test, and differences were considered significant when *P* was <0.05.

## RESULTS

**Toxicity.** All three Cry1A toxins had similar insecticidal activities. The LC<sub>50</sub> measured for Cry1Aa, the most toxic of the

TABLE 1. Effect of *B. thuringiensis* toxin concentrations on the mortality and weight gain of *M. sexta* larvae<sup>a</sup>

Toxin	LC <sub>50</sub> (ng/cm <sup>2</sup> )	Slope ± SEM for LC <sub>50</sub>	ED <sub>50</sub> <sup>b</sup> (ng/cm <sup>2</sup> )	Slope ± SEM for ED <sub>50</sub>
Cry1Aa	27 (25–29)	3.4 ± 0.3	2.9 (2.5–3.4)	1.3 ± 0.2
Cry1Ab	39 (36–42)	2.7 ± 0.1	14.6 (13.7–15.6)	3.9 ± 0.3
Cry1Ac	34 (32–37)	3.2 ± 0.2	3.7 (3.3–4.1)	2.3 ± 0.2
Cry1Ca	103 (97–109)	2.7 ± 0.2	26 (25–28)	3.7 ± 0.6
Cry1Ea	78 (73–83)	2.85 ± 0.08	28 (26–30)	2.9 ± 0.4
R233A	144 (135–154)	3.6 ± 0.1	ND <sup>c</sup>	ND

<sup>a</sup> 95% confidence intervals are shown in parentheses.

<sup>b</sup> ED<sub>50</sub>, 50% weight gain inhibitory dose.

<sup>c</sup> ND, not determined.

three, was more than one-half that measured for Cry1Ab, the least toxic (Table 1). On the other hand, the LC<sub>50</sub> values measured for Cry1Ca, Cry1Ea, and R233A were, respectively, approximately 3.8, 2.9, and 5.3 times higher than that measured for Cry1Aa. However, the toxin concentrations necessary to inhibit weight gain by 50% were similar for Cry1Aa and Cry1Ac but approximately 5 times higher for Cry1Ab, 9 times higher for Cry1Ca, and 10 times higher for Cry1Ea than for Cry1Aa (Table 1). Weight gain by *M. sexta* larvae was inhibited almost completely by 1,000 ng R233A/ml (55 ng/cm<sup>2</sup>), the lowest concentration of this toxin that was tested (data not shown).

**Effect of *B. thuringiensis* toxins on the membrane potential of *M. sexta* midgut epithelial cells.** The activity of Cry toxins was studied in midguts isolated from third-instar *M. sexta* larvae with an electrophysiological procedure developed earlier in our laboratory (30). The initial membrane potential was -67 ± 2 mV (mean ± standard error of the mean [SEM] of 125 measurements). As was observed previously (11, 30), in the absence of toxin, membrane potential decreased when the perfusion was stopped but returned to its original value soon after the perfusion was resumed (Fig. 1A). When tested at a concentration of 10 µg/ml, all toxins (Fig. 1) depolarized the membrane efficiently except Cry1Ba (Fig. 1E), which has little toxicity for *M. sexta* (12, 14). At 1 µg/ml, Cry1Ac (Fig. 1D), Cry1Ca (Fig. 1F), and Cry1Ea (Fig. 1G) depolarized the membrane efficiently, with PR<sub>65</sub> values (7 ± 2, 4 ± 1, and 2.8 ± 0.4 min, respectively) that did not differ significantly from each other. However, Cry1Ab was significantly less efficient, with a PR<sub>65</sub> of 12 ± 1 min (Fig. 1C), whereas both Cry1Aa and its mutant, R233A, had little effect on membrane potential at this concentration (Fig. 1B and H).

**Effect of midgut juice on the activity of *B. thuringiensis* toxins.** Midgut juice composition was first determined to evaluate the influence of adding 10% midgut juice during the incubation period with the toxin on the KCl concentration and the osmolality of the bathing solution. Midgut juice contained 1.88 ± 0.01 mM Na<sup>+</sup>, 230.4 ± 0.5 mM K<sup>+</sup>, and 19 ± 1 mM Cl<sup>-</sup> and had an osmolality of 367 ± 2 mOsm/kg (means ± SEM for three different samples). For experiments performed with 10% midgut juice, the 122K solution was therefore replaced by a solution containing 110 mM potassium but having an otherwise similar composition, in order to achieve a final potassium concentration of 122 mM. This replacement had a negligible effect on the osmolality of the bathing solution. In the presence of

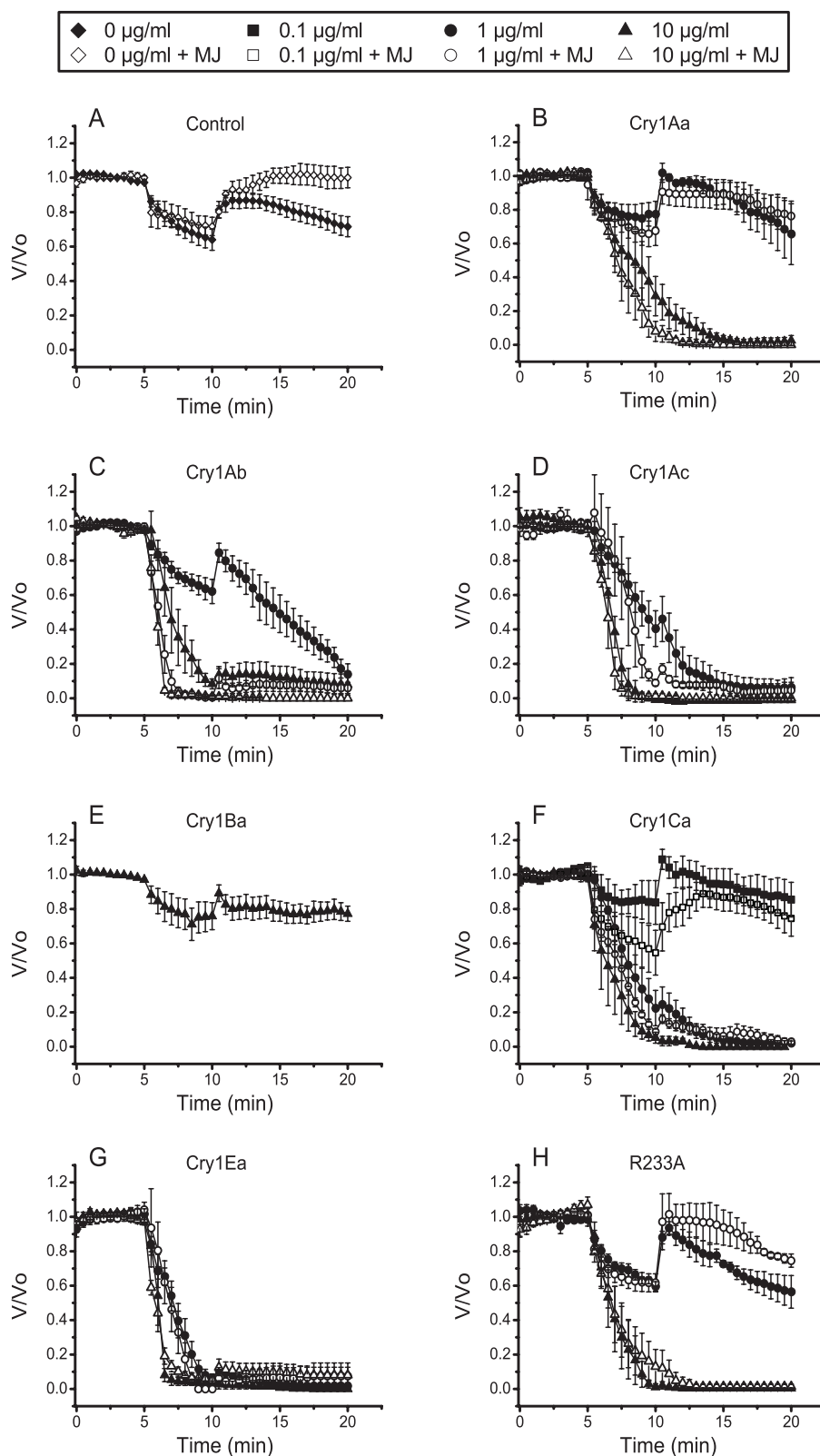


FIG. 1. Effect of midgut juice on the ability of *B. thuringiensis* toxins to depolarize the luminal membrane of epithelial cells of isolated *M. sexta* midguts. The bath was perfused with the 122K solution until membrane potential was stable over 5 min. Perfusion was then stopped, and 1 ml of the 122K solution containing no toxin (A), Cry1Aa (B), Cry1Ab (C), Cry1Ac (D), Cry1Ba (E), Cry1Ca (F), Cry1Ea (G), or the Cry1Aa mutant R233A (H), with ( $\diamond$ ,  $\square$ ,  $\circ$ ,  $\triangle$ ) or without ( $\blacklozenge$ ,  $\blacksquare$ ,  $\bullet$ ,  $\blacktriangle$ ) 10% (vol/vol) midgut juice (MJ), was added directly to the bath. The toxin concentrations used were 0, 0.1, 1, or 10  $\mu\text{g/ml}$ . After 5 min, the preparation was rinsed with the 122K solution for 10 min. Data are means  $\pm$  SEM for three to eight independent experiments.

10% midgut juice, the pH of the solution was  $9.75 \pm 0.09$  (mean  $\pm$  SEM for five different samples). Based on previous studies (36, 38), the activities of the toxins tested in the present study are expected to be similar at this pH and at pH 10.5. Furthermore, adding 10% midgut juice had little effect on the membrane potential measured in the absence of toxin (Fig. 1A). Membrane potential was also unaffected by this addition when measured in the presence of Cry1Aa (Fig. 1B), Cry1Ac (Fig. 1D), Cry1Ca (Fig. 1F), Cry1Ea (Fig. 1G), or R233A (Fig. 1H) at all toxin concentrations tested. In contrast, midgut juice had a major effect on the rate of membrane depolarization by Cry1Ab at 1  $\mu\text{g}/\text{ml}$  (Fig. 1C). Under these conditions,  $\text{PR}_{65}$  was reached after  $1.4 \pm 0.2$  min, a rate which was slightly more rapid than those measured for Cry1Ac ( $3.3 \pm 0.5$  min) (Fig. 1D), Cry1Ca ( $2.9 \pm 0.2$  min) (Fig. 1F), and Cry1Ea ( $2.4 \pm 0.3$  min) (Fig. 1G), even though the difference was significant only when Cry1Ab was compared with Cry1Ac and Cry1Ca.

The cause of this effect of midgut juice on the activity of Cry1Ab was further investigated by testing the toxin in the presence of a cocktail of protease inhibitors, with or without midgut juice, or in the presence of midgut juice which had been boiled for 5 min. In the absence of toxin, the midgut juice, boiled or not, as well as the protease inhibitor cocktail, with or without midgut juice, had no significant effect on membrane potential (Fig. 2A). However, the effect of midgut juice on the depolarization of the membrane by 1  $\mu\text{g}$  Cry1Ab/ml was slightly reduced when it was replaced by boiled midgut juice ( $\text{PR}_{65} = 3.16 \pm 0.06$  min) (Fig. 2B). In the absence of midgut juice, the cocktail of protease inhibitors enhanced the activity of Cry1Ab ( $\text{PR}_{65} = 6 \pm 1$  min). In the presence of midgut juice and the cocktail, the membrane depolarization induced by Cry1Ab ( $\text{PR}_{65} = 2.2 \pm 0.4$  min) was similar to that observed in the presence of midgut juice only.

## DISCUSSION

To test the hypothesis that midgut proteases could enhance or inhibit toxin activity, the effect of midgut juice on the activity of Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, Cry1Ea, and R233A was studied in midguts isolated from third-instar *M. sexta* larvae using a microelectrode technique (30). As mentioned elsewhere (16), this technique, in contrast with the light scattering assay using brush border membrane vesicles (37), allows the study of toxin activity in the presence of midgut juice. In parallel, each of these toxins was evaluated for its effect on the mortality and weight gain of larvae of the same species.

The toxicities evaluated in the present study (Table 1) were similar to those of Van Rie et al. (40, 41), who reported  $\text{LC}_{50}$  and 95% confidence interval values (parentheses) for Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, and Cry1Ea of, respectively, 20 (15 to 29), 20 (15 to 28), 9 (6 to 12), 111 (76 to 163), and 73 (46 to 115)  $\text{ng}/\text{cm}^2$ . However, Cry1Ac, which was slightly more toxic to *M. sexta* than Cry1Aa and Cry1Ab in previous studies (7, 40), was slightly less toxic than Cry1Aa (Table 1). On the other hand, although Coux et al. (7) observed lower  $\text{LC}_{50}$  values for Cry1Aa (3.1 [2.5 to 3.7]  $\text{ng}/\text{cm}^2$ ) and Cry1Ac (1.65 [1.45 to 1.8]  $\text{ng}/\text{cm}^2$ ) than those found in the present study, the activity of the Cry1Aa mutant that they observed was closer to that measured in the present study. They measured  $28 \pm 1$  percent mortality for the Cry1Aa mutant R233A at 100  $\text{ng}/\text{cm}^2$ ,

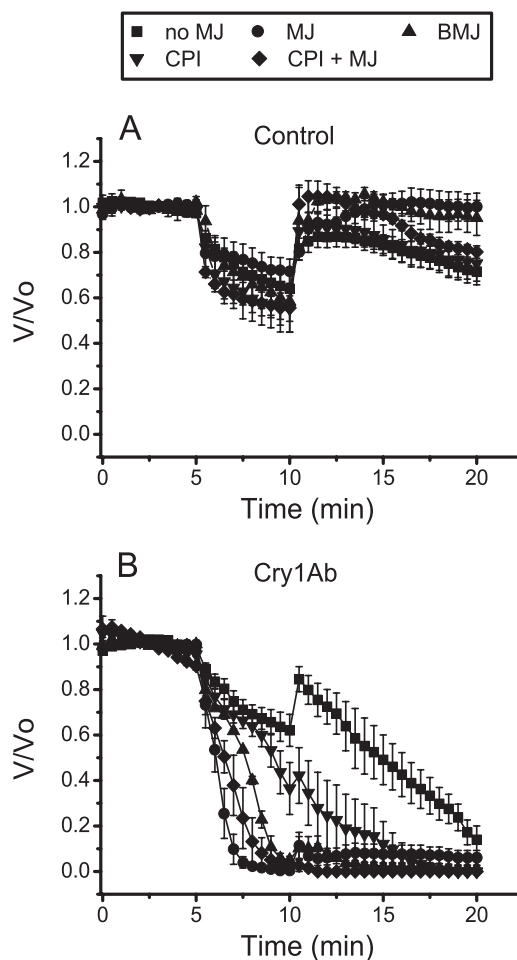


FIG. 2. Effect of midgut juice and protease inhibitors on the activity of Cry1Ab. The bath was perfused with the 122K solution until membrane potential was stable over 5 min. Perfusion was then stopped, and 1 ml of the 122K solution containing either no toxin (A) or 1  $\mu\text{g}/\text{ml}$  of Cry1Ab (B) alone (■) or with either the cocktail of protease inhibitors (1% [vol/vol]) (CPI) (▼), 10% midgut juice (MJ) with (◆) or without (●) the cocktail of protease inhibitor (1% [vol/vol]) or 10% (vol/vol) boiled midgut juice (BMJ) (▲) was added. Data are means  $\pm$  SEM for three to eight independent experiments.

whereas, in the present study,  $34 \pm 7$  percent mortality was observed for R233A at 109.5  $\text{ng}/\text{cm}^2$ . In addition, Gilliland et al. (12) found  $\text{LC}_{50}$  values for Cry1Ac that were approximately 27 and 4.5 times lower than those obtained for Cry1Ca when testing neonate and third-instar larvae, respectively.

All toxins at 10  $\mu\text{g}/\text{ml}$ , except Cry1Ba, which has little toxicity for *M. sexta* (12, 14), depolarized the membrane efficiently in the presence or absence of midgut juice. However, the fine details of the pattern of toxicity should be better reflected in the electrophysiological experiments when the toxins are tested at 1  $\mu\text{g}/\text{ml}$ . In the absence of midgut juice, at 1  $\mu\text{g}/\text{ml}$ , Cry1Aa, which was the most toxic in this study, had little effect on the membrane potential while Cry1Ab, the least toxic of the Cry1A toxins, depolarized the membrane slowly but almost completely within the time course of the experiment and Cry1Ac depolarized the membrane efficiently and rapidly. Furthermore, Cry1Ca and Cry1Ea, despite having a lower toxicity,



depolarized the membrane as efficiently and as rapidly as Cry1Ac. The lower activity of Cry1Aa and Cry1Ab is rather surprising, in view not only of the *in vivo* toxicity but also of the previous *in vitro* studies. Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Ea had similar pore-forming activities in brush border membrane vesicles, as observed using an osmotic-swelling assay (36) or a potential-sensitive fluorescent dye (17). The lower pore-forming ability estimated for Cry1Ca using the osmotic-swelling assay (36) compared with that measured using the potential-sensitive dye (17) was explained by the effect of ionic strength on the activity of this toxin (11). The rapid depolarization observed for Cry1Ea in the present study correlates well with its strong pore-forming ability in brush border membrane vesicles (36).

Although midgut juice had little or no effect on the activity of Cry1Aa, Cry1Ac, Cry1Ca, Cry1Ea, or the R233A mutant, it greatly increased the membrane depolarization ability of Cry1Ab. Different components of the midgut juice could contribute to this enhanced activity, but midgut proteases do not appear to be involved since adding the cocktail of protease inhibitors did not affect significantly the combined effect of the midgut juice and Cry1Ab. Furthermore, boiling the midgut juice induced only a small, albeit significant, reduction of its capacity to enhance Cry1Ab activity. The contribution of proteins to the effect of midgut juice on Cry1Ab activity therefore appears rather modest. Further studies will obviously be necessary to elucidate the mechanism underlying this stimulation and the reasons why it occurs specifically for Cry1Ab and not for the other toxins tested. On the other hand, the cocktail of protease inhibitors enhanced the activity of Cry1Ab measured in the absence of midgut juice. This effect is most probably due to the inhibition of membrane-bound proteases rather than to an increase in membrane permeability resulting from the presence of the inhibitors since these compounds had no detectable effect on membrane potential when measured in the absence of added toxin (Fig. 2A) or on the permeability of brush border membrane vesicles (16). Thus, in contrast with earlier suggestions (5, 13, 20, 29), cleavage within the trypsin-activated Cry1Ab molecules by membrane proteases or midgut juice proteases does not appear to be necessary for or to stimulate toxin activity. On the contrary, membrane-bound proteases appear to inhibit Cry1Ab activity. This observation contrasts somewhat with our previous finding that the pore-forming ability of Cry1Aa in *M. sexta* midgut brush border membrane vesicles is unaffected by protease inhibitors (16). It should be pointed out, however, that the enzymatic activity is expected to be much better preserved in the freshly isolated midguts used in the present study than in vesicles that were prepared from frozen midguts using a much lengthier protocol. In any case, both studies agree in concluding that the activated toxin does not require further proteolytic processing to become functional. On the other hand, the absence of effect of midgut juice on the activity of R233A demonstrates that its lower toxicity, despite a pore-forming activity which was similar to that of Cry1Aa in brush border membrane vesicles (7) and in isolated midguts (Fig. 1), cannot be explained by its increased susceptibility to proteolysis in the insect midgut.

The *in vivo* toxicity of *B. thuringiensis* toxins (Table 1) correlates only partially with their *in vitro* activity, estimated from their ability to abolish the apical membrane potential of the

insect midgut (Fig. 1) or from their ability to increase the permeability of vesicles prepared from this membrane (17, 36). In the electrophysiological assay, the pore-forming ability of the toxins is measured in living cells in which the presence of a strong membrane potential, which is absent during the incubation of the vesicles with the toxin prior to the osmotic swelling (36) and the fluorescence assay (17), could influence pore formation. However, this possibility does not appear to be sufficient to explain why the relative toxicity and *in vitro* activity of the toxins do not follow a similar pattern. These differences could possibly result, at least in part, from the fact that the brush border membrane vesicles (17, 36) and midgut juice were isolated from fifth-instar larvae, the electrophysiological experiments were carried with midguts isolated from third-instar larvae, and *in vivo* toxicity data were obtained using neonate larvae. Numerous authors have reported differential effects of the larval instar on the potency of *B. thuringiensis* toxins (2, 3, 9, 12, 18, 19, 21, 24, 31, 39, 42). For example, Gilliland et al. (12) observed that *M. sexta* third-instar larvae had significantly fewer binding sites for Cry1Ac than neonates while no significant changes were observed for Cry1Ca. Similarly, Rausell et al. (31) reported that a binding site for Cry1Ab is lost from last-instar larvae of *Thaumetopoea pityocampa* and *Lymantria monacha*. These observations suggest that the effect of larval instar on the *in vitro* pore-forming activity of Cry toxins should be examined. Considering the difficulties inherent to the preparation of brush border membrane vesicles from small insect larvae, the electrophysiological assay used in the present study should provide a method of choice for such experiments.

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